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CADMIUM AND ZINC TOXICITY TO JORDANELLA FLORIDAE

by

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FOREWORD

Our nation's freshwaters are vital for all animals and plants, yet our diverse uses of water---for recreation, food, energy, transportation, and industry---physically and chemically alter lakes, rivers, and streams. Such alterations threaten terrestrial organisms, as well as those living in water. The Environmental Research Laboratory in Duluth, Minnesota develops methods, conducts laboratory and field studies, and extrapolates research findings

--to determine how physical and chemical pollution affects aquatic life

--to assess the effects of ecosystems on pollutants

--to predict effects of pollutants on large lakes through use of models

--to measure bioaccumulation of pollutants in aquatic organisms that are consumed by other animals, including man

This report deals with two chemical pollutants, cadmium and zinc, and their effects on the flagfish (Jordanella floridae). These chemicals were utilized because they are virtually ubiquitous in the environment and because of their observed toxic effect to some aquatic life. The flagfish was chosen for study because of its short life cycle (6-8 wk) and for comparing its sensitivity with other fish species having longer life cycles.

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ABSTRACT

Cadmium and zinc toxicity to the flagfish (Jordanella floridae) was determined on the basis of 96-hr median lethal concentrations (LC50) and significant decreases (P = 0.05) in survival, growth, and reproduction over the complete life cycle of the fish. The 96-hr LC50 values for cadmium and zinc to juvenile flagfish were 2,500 and 1,500 µg/liter, respectively. In chronic tests, reproduction was the most sensitive indicator of cadmium toxicity and was inhibited at 8.1 µg/liter. Tissue-concentration analysis showed that fish exposed to concentrations of 1.7 µg/liter and above accumulated significantly greater amounts of cadmium than those in the controls. In zinc tests, survival of larvae (not exposed as embryos) and growth of females were the most sensitive measure of zinc toxicity and were reduced at respective concentrations of 85 and 51 µg/liter. Significant uptake of zinc occurred in fish exposed to concentrations of 47 µg/liter and above. The lowest cadmium and zinc concentrations causing adverse effects to the flagfish were similar to those affecting other fish species. Application factors for both metals were similar to those reported for cadmium exposed bluegills (Lepomis macrochirus) and zinc exposed fathead minnows (Pimephales promelas) in hard water.

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CONTENTS

	Page
Foreword	iii
Abstract	iv
Acknowledgments	vi
1. Introduction	1
2. Conclusions	2
3. Recommendations	3
4. Materials and Methods	4
Water characteristics	4
Exposure system	4
Toxicant solution	4
Tissue analysis	8
Biological procedures	8
Statistical analysis	9
5. Results	10
Cadmium toxicity	10
Zinc toxicity	12
Resulting test values and application factors	12
6. Discussion	14
References	16
Appendices	
A. Recommended bioassay procedure for *Jordanella floridae* (Goode and Bean) chronic tests	19
B. Cadmium concentrations in whole fish tissue after 30 and 100 days of exposure	32
C. Zinc concentrations in whole fish tissue after 30 and 100 days of exposure (test 1 begun with larvae exposed as embryos; test 2 begun with unexposed larvae)	33

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SECTION 1

INTRODUCTION

Cadmium and zinc occur simultaneously in the environment (Lingane 1966; Schroeder et al. 1967) and are toxic to fish (National Academy of Sciences 1973). Although most toxicity tests with fish have been short-term acute exposures, an effort is being made at the Environmental Research Laboratory-Duluth to determine more sensitive effects of toxicants through chronic exposures of fish over their complete life cycle. Commonly used species, such as fathead minnows (Pimephales promelas), bluegills (Lepomis macrochirus), brook trout (Salvelinus fontinalis), and rainbow trout (Salmo gairdneri), however, require long testing periods (5 months to 3 years) to obtain complete life cycle data. Smith (1973) has proposed the use of the flagfish (Jordanella floridae) for rapid chronic bioassays because of its short generation time (6-8 weeks) and other unique features. Little information has been shown on the effects of toxicants on this species in the literature (Foster et al. 1966, 1969).

The purpose of this work was to determine the toxicity of cadmium and zinc to the flagfish by studying the effects of these metals on all developmental stages of the life cycle. In addition, this study was designed to evaluate the application factor concept (Mount and Stephan 1967b) with a species not previously tested.

SECTION 2

CONCLUSIONS

1. Reproduction was the most sensitive indicator of cadmium toxicity and was inhibited at 8.1 µg/liter.

2. Significant cadmium uptake occurred in fish exposed to concentrations of 1.7 µg/liter and above. Uptake increased with increasing exposure concentrations but leveled off at 16 µg/liter indicating an equilibrium between tissue and water concentrations. Uptake also increased with time but at a rate that was slower than the growth of the fish.

3. Survival of larvae (not exposed as embryos) and growth of females were the most sensitive measure of zinc toxicity and were reduced at respective concentrations of 85 and 51 µg/liter.

4. Significant zinc uptake occurred in fish exposed to concentrations of 47 µg/liter and above. Zinc uptake increased with increasing exposure concentrations and increased with time, but at a rate that was slower than the growth of the fish.

5. The lowest cadmium and zinc concentrations causing adverse effects to the flagfish were similar to those affecting other fish species.

6. Flagfish application factors for both metals were similar to those reported for cadmium exposed bluegills and zinc exposed fathead minnows in hard water.

SECTION 3

RECOMMENDATIONS

Concentrations of cadmium and zinc not exceeding 4 and 26 µg/liter, respectively, appear "safe" for the flagfish and are suggested as maximum permissible levels under the conditions tested. However, since significant cadmium uptake occurred in fish exposed to 1.7 µg/liter, maximum permissible levels for cadmium may be lower in longer term exposures than were conducted in this study. Additional tests in waters of various qualities are also needed to more fully define the no-effect levels for these metals in natural waters.

Further testing with this species and other toxic substances is recommended for the determination of water quality criteria.

SECTION 4

MATERIALS AND METHODS

WATER CHARACTERISTICS

Untreated Lake Superior water was used in all tests at 25 ± 2 C. Chemical characteristics of the test water were analyzed weekly according to methods described by the American Public Health Association et al. (1971). The means and ranges (in parentheses) for these measurements were (mg/liter): Dissolved oxygen, 8.3 (7.2-9.5); hardness, 44 as $CaCO_3$ (41-46); alkalinity, 42 as $CaCO_3$ (39-44); and acidity, 2.4 as $CaCO_3$ (1.5-3.4). The pH ranged from 7.1 to 7.8.

EXPOSURE SYSTEM

The exposure system consisted of a proportional diluter (Mount and Brungs 1967) which delivered five toxicant concentrations and a control to duplicate exposure chambers. Spawning chambers were glass aquaria, 30- x 60- x 30-cm, with a water volume of 43 liters. Larval chambers were 15- x 53- x 22-cm and contained 22 liters of water. Flow rate to each spawning chamber was 15 liter/hr providing 95% replacement of test water every 9 hr (Sprague 1969).

A combination of Duro-Test (Optima FS) and wide spectrum Gro-Lux fluorescent bulbs provided a light intensity of 50 lumens at the water surface. An automatically controlled 16 hr photoperiod was used.

TOXICANT SOLUTION

The stock solutions were prepared by dissolving reagent-grade $CdCl_2$ and $ZnSO_4 \cdot 7H_2O$ in 15 liters of distilled water and were introduced from a Mariotte bottle to the diluter by a chemical metering device (McAllister et al. 1972). Weekly composite samples of the test water were analyzed by a Perkin Elmer Model 403 atomic absorption spectrophotometer. Measurements for cadmium concentrations in the water are included in Table 1 and those for two zinc tests are shown in Tables 2 and 3. Analysis of variance showed that zinc measurements were the same for the two tests. The method of known additions of cadmium and zinc to Lake Superior (control) water was used to construct calibration curves. The mean percent recovery and standard deviation for 16 spiked cadmium and zinc samples were 91 ± 0.8 and 100 ± 2.0, respectively.

TABLE 1. SURVIVAL, GROWTH, AND REPRODUCTION OF FLAGFISH EXPOSED TO SEVERAL CONCENTRATIONS OF CADMIUM

	Measured cadmium concentration (µg/liter)											
	31 ± 5.6[a]		16 ± 2.9		8.1 ± 2.0		4.1 ± 0.81		1.7 ± 0.52		0.11 ± 0.07 (control)	
	A[b]	B[b]	A	B	A	B	A	B	A	B	A	B
					30 days							
Survival (%)[c]	70	10	77	77	77	97	87	93	97	100	97	97
Mean total length (mm)	16 ± 1.5[a] *	--	19 ± 1.6	18 ± 2.3	18 ± 0.7	20 ± 1.7	20 ± 1.9	18 ± 2.3	18 ± 3.5	17 ± 2.1	20 ± 1.0	19 ± 1.7
					100 days							
Males/females at termination	0/0	0/1	0/3	0/1	2/5	2/5	2/5	2/5	2/5	2/5	2/5	2/5
Mean total length (mm)												
Male	--		--		49 ± 7.8 (4)		53 ± 1.7 (4)		52 ± 1.7 (4)		55 ± 3.3 (4)	
Female	31 (1)[d] *		33 ± 2.1 (4) *		43 ± 3.2 (10)		43 ± 3.1 (10)		43 ± 2.2 (10)		45 ± 2.9 (10)	
Mean spawnings/female	0 *	0	1.7 *	1.0	5.2 *	3.0	10.4	8.6	11.0	11.4	8.8	8.4
Total embryos produced	0 *	0	163 *	35	1,560 *	1,270	3,320	3,280	4,240	3,440	3,220	3,430
Mean hatchability (%)	—		68		66		73		66		66	

[a]Mean ± standard deviation.

[b]Duplicate chamber.

[c]Thirty fish per chamber.

[d]Number of fish analyzed.

*Significantly different from control according to Dunnett's procedure (P = 0.05).

TABLE 2. SURVIVAL, GROWTH, AND REPRODUCTION OF FLAGFISH (initially exposed as embryos) AT SEVERAL CONCENTRATIONS OF ZINC (test 1)

	Measured zinc concentration (µg/liter)											
	267 ± 28[a]		139 ± 18		75 ± 11		47 ± 11		28 ± 11		10 ± 13 (control)	
	A[b]	B[b]	A	B	A	B	A	B	A	B	A	B
					30 days							
Survival (%)[c]	10	0	70	80	100	97	90	100	83	90	83	97
Mean total length (mm)	--	-	18 ± 2.2[a]	18 ± 0.9	18 ± 2.0	18 ± 1.4	19 ± 1.7	19 ± 1.8	18 ± 2.7	19 ± 2.0	18 ± 2.5	19 ± 2.0
					100 days							
Males/females at termination	0/0	0/0	0/4	2/5	2/5	2/5	2/5	2/5	2/5	2/5	2/5	2/5
Mean total length (mm)												
Male	--	--	45 ± 5.7 (2)[d*]		51 ± 4.5 (4)		55 ± 2.7 (4)		56 ± 3.4 (4)		54 ± 3.4 (4)	
Female	--	--	40 ± 6.0 (9)		43 ± 2.5 (10)		43 ± 1.9 (10)		43 ± 2.4 (10)		43 ± 2.2 (10)	
Mean spawnings/female	--	--	0.3	1.4	4.0	4.6	9.4	3.4	4.8	4.4	10.8	4.8
Total embryos produced	--	--	130	197	1,310	1,650	3,230	995	1,560	1,240	3,650	1,190
Mean hatchability	--		73		59		72		76		70	

[a]Mean ± standard deviation.

[b]Duplicate chamber.

[c]Thirty fish per chamber.

[d]Number of fish analyzed.

*Significantly different from control according to Dunnett's procedure (P = 0.05).

TABLE 3. SURVIVAL, GROWTH, AND REPRODUCTION OF FLAGFISH (not exposed as embryos) AT SEVERAL CONCENTRATIONS OF ZINC (test 2)

	Measured zinc concentrations (µg/liter)											
	267 ± 28[a]		139 ± 18		85 ± 11		51 ± 9		26 ± 8		<1.0 (control)	
	A[b]	B[b]	A	B	A	B	A	B	A	B	A	B
30 days												
Survival (%)[c]	0	0	0	0	20 *	23 *	82	97	93	93	93	87
Mean total length (mm)	-	-	-	-	--	--	22 ± 2.3[d]	21 ± 3.5	21 ± 1.7	22 ± 1.8	23 ± 2.4	22 ± 2.4
100 days												
Males/females at termination	0/0	0/0	0/0	0/0	2/3	3/4	2/5	2/5	2/5	2/5	2/5	2/5
Mean total length (mm)												
Male	-	-	-	-	52 ± 8.4 (5)[d]		53 ± 2.9 (4)		53 ± 1.7 (4)		54 ± 1.8 (4)	
Female	-	-	-	-	36 ± 4.6 (7) *		38 ± 5.5 (10) *		40 ± 3.5 (10)		43 ± 2.5 (10)	
Mean spawnings/female	-	-	-	-	4.0	0.3	2.2	6.6	5.4	3.2	9.4	4.6
Total embryos produced	-	-	-	-	322	11	272	2,440	1,050	348	2,320	1,360
Mean hatchability (%)	-		-		88		83		83		69	

[a] Mean ± standard deviation.

[b] Duplicate chamber.

[c] Thirty fish per chamber.

[d] Number of fish analyzed.

* Significantly different from control according to Dunnett's procedure (P = 0.05).

TISSUE ANALYSIS

Whole fish from each concentration were analyzed for cadmium and zinc after 30 days of exposure and at the end of the test (approximately 100 days). For the cadmium test, 10 fish were analyzed per concentration except at 16 and 31 µg/liter where only one and four fish survived, respectively. Five fish were analyzed per concentration in each of the two zinc tests. A 95 to 100% recovery of the metals for 35 spiked tissue samples was obtained using a wet digestion, atomic absorption spectrophotometric analysis (Leonard 1971).

BIOLOGICAL PROCEDURES

In chronic tests, enough embryos were incubated in each exposure concentration (cadmium test, zinc test 1) or in the controls (zinc test 2) to provide 30 1-day-old larvae to each chamber for the start of the tests. Both exposed larvae and larvae not previously exposed as embryos were utilized for zinc exposures to determine the effect of embryo acclimation on larval sensitivity. All embryos were treated with metal-free malachite green (4 mg/liter) for 10 min during the first 3 days of incubation to control fungus.

Larvae were fed brine shrimp nauplii four times a day for the first 30 days. After this time, all fish were randomly reduced to 15 per chamber and were fed a diet of frozen brine shrimp twice a day and salmon starter granules once a day. The number of fish per chamber was further reduced to 2 males and 5 females after males began displaying territorial behavior, about the 60th day of the test.

Reproduction studies were started after 30 or more embryos appeared on any one spawning substrate and were continued for a period of 45 days. Spawning substrates were approximately 13-cm long by 8-cm wide and were made from 20 mesh stainless steel screen that was corregated and wrapped in dark orlon yarn. After spawning, 30 to 50 embryos were removed from the substrates, placed in oscillating egg cups (Mount 1968) and incubated in the test water. All embryos not incubated for hatchability determinations were counted and discarded.

Twenty newly hatched (F_1) larvae from each toxicant concentration were randomly selected and transferred to duplicate larval chambers for 30 day growth and survival exposure. In the cadmium test, control larvae were also transferred to concentrations causing adverse effects to parental fish, for 30 days. Additional procedures were done in accordance with the bioassay procedures for Jordanella floridae recommended by the Environmental Research Laboratory-Duluth (U.S. Environmental Protection Agency 1972) (Appendix A).

Flow-through 96-hr acute toxicity tests were conducted in the chronic test diluter system with juvenile flagfish 4-5 weeks old, according to methods described by the American Public Health Association et al. (1971). The 96-hr LC50 (median lethal concentration) values were derived by

graphical interpolation and were used in the determination of application factors (maximum acceptable toxicant concentration (MATC)/96-hr LC50) (Mount and Stephan 1967b) for both metals.

STATISTICAL ANALYSIS

For statistical evaluation, survival, growth, reproduction, and cadmium tissue accumulation data were subjected to an analysis of variance ($P = 0.05$) and Dunnett's two tailed comparison of treatment means to control means ($P = 0.05$) (Steel and Torrie 1960). A two way analysis of variance was applied to zinc accumulation data for comparisons between tests.

SECTION 5

RESULTS

CADMIUM TOXICITY

In 30 days, the highest concentration (31 µg/liter) retarded growth and appeared to reduce survival (Table 1). Survival also appeared reduced at the next lower concentration (16 µg/liter). However, due to the variability of response in duplicate chambers, statistical significant differences ($P = 0.05$) in survival could not be shown in any of the concentrations. Additional mortality occurred at 16 and 31 µg/liter after this period and by the 60th day, survival was less than 35% and growth of fish at 16 µg/liter appeared to be reduced. Cadmium-induced mortalities of fish were always preceded by periods of involuntary muscle spasms. At the end of the test (approximately 100 days) all males were dead in the two highest concentrations and growth of five surviving females was significantly reduced. Mean spawnings/female and embryo production was adversely affected at 8.1 µg/liter (Table 1). No significant differences in survival, growth, or reproduction were observed below 8.1 µg/liter.

Embryos from exposed parents incubated at 16 µg/liter and below hatched as well as those in the control (Table 1). Survival and growth of F_1 larvae were not adversely affected at these concentrations after 30 days of exposure; however, survival of larvae transferred at hatch from control water to 31 µg/liter was less than 20%. No adverse effects were seen when control larvae were transferred to 16 µg/liter and below for this period.

The 96-hr LC50 value calculated for 4-5 week old juveniles was 2,500 µg Cd/liter.

Fish exposed for 30 days to concentrations of 1.7 µg Cd/liter and above contained significantly higher amounts of cadmium than did those in the controls (Figure 1). Cadmium uptake increased with increasing exposure concentrations but leveled off at 16 µg/liter. Concentrations of cadmium (µg/gram) were lower in fish exposed for 100 days than those exposed for 30 days but older fish contained a significantly higher total body content (µg/fish). In addition, cadmium concentrations in 30-day-old exposed parental fish and progeny (F_1) were similar (Appendix B).

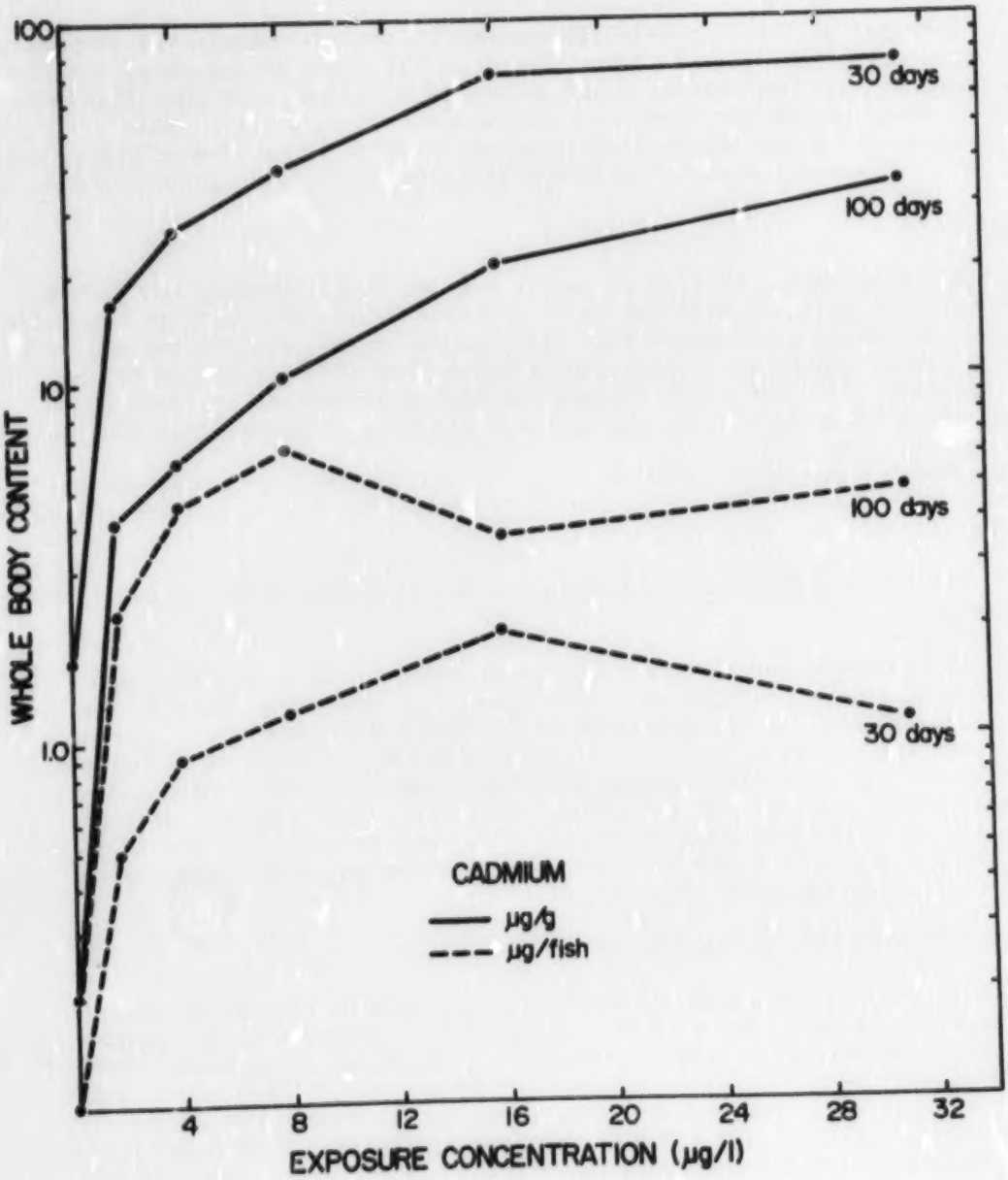

Figure 1. Log cadmium content measured from whole body tissue of flagfish exposed to several cadmium concentrations for 30 and 100 days.

ZINC TOXICITY

Test 1

Survival of larvae previously exposed to zinc as embryos was reduced at 267 µg/liter after 30 days (Table 2). After 100 days of exposure, all fish had died at this concentration and growth of males was less than those of the controls at the next lower concentration (139 µg/liter). Mean spawnings/female and embryo production appeared to be reduced at 139 µg/liter but significant differences could not be shown due to the variable response.

Test 2

After 30 days, survival of larvae not previously exposed to zinc as embryos was adversely affected at 85 µg/liter (Table 3). No larvae survived at the two highest concentrations (139 and 267 µg/liter). By the end of the test, female growth was significantly lower than the controls at 85 and 51 µg/liter. Mean spawnings/female and embryo production appeared to be reduced at 85 µg/liter but were not statistically different from the controls.

Embryo hatchability (Tables 2 and 3) and survival and growth of F_1 larvae from exposed parents at 139 µg/liter and below were not significantly different from those of control larvae.

The 96-hr LC50 value calculated for 4-5 week old juveniles was 1,500 µg Zn/liter.

Fish exposed for 30 days to concentrations of 47 µg Zn/liter and above contained significantly higher amounts of zinc than those in the controls (Figure 2). Additionally, zinc uptake increased with increasing exposure concentrations. Concentrations of zinc (µg/gram) in fish exposed for 30 and 100 days were similar but older fish contained a significantly higher total body content (µg/fish). Zinc concentrations were also similar in 30-day-old exposed parental fish and progeny (F_1) and in fish previously exposed as embryos (test 1) and those not previously exposed (test 2) after 100 days (Appendix C).

RESULTING TEST VALUES AND APPLICATION FACTORS

The results described above for chronic effects of cadmium and zinc on survival, growth, and reproduction establish the maximum acceptable toxicant concentration [(MATC) as described by Mount and Stephan (1967b)] between 4.1 and 8.1 µg/liter cadmium and 26 and 51 µg/liter zinc. The application factor (MATC/96-hr LC50) based on the 96-hr LC50 values of 2,500 µg Cd/liter and 1,500 µg Zn/liter was between 0.0016 and 0.0032 for cadmium and 0.017 and 0.034 for zinc.

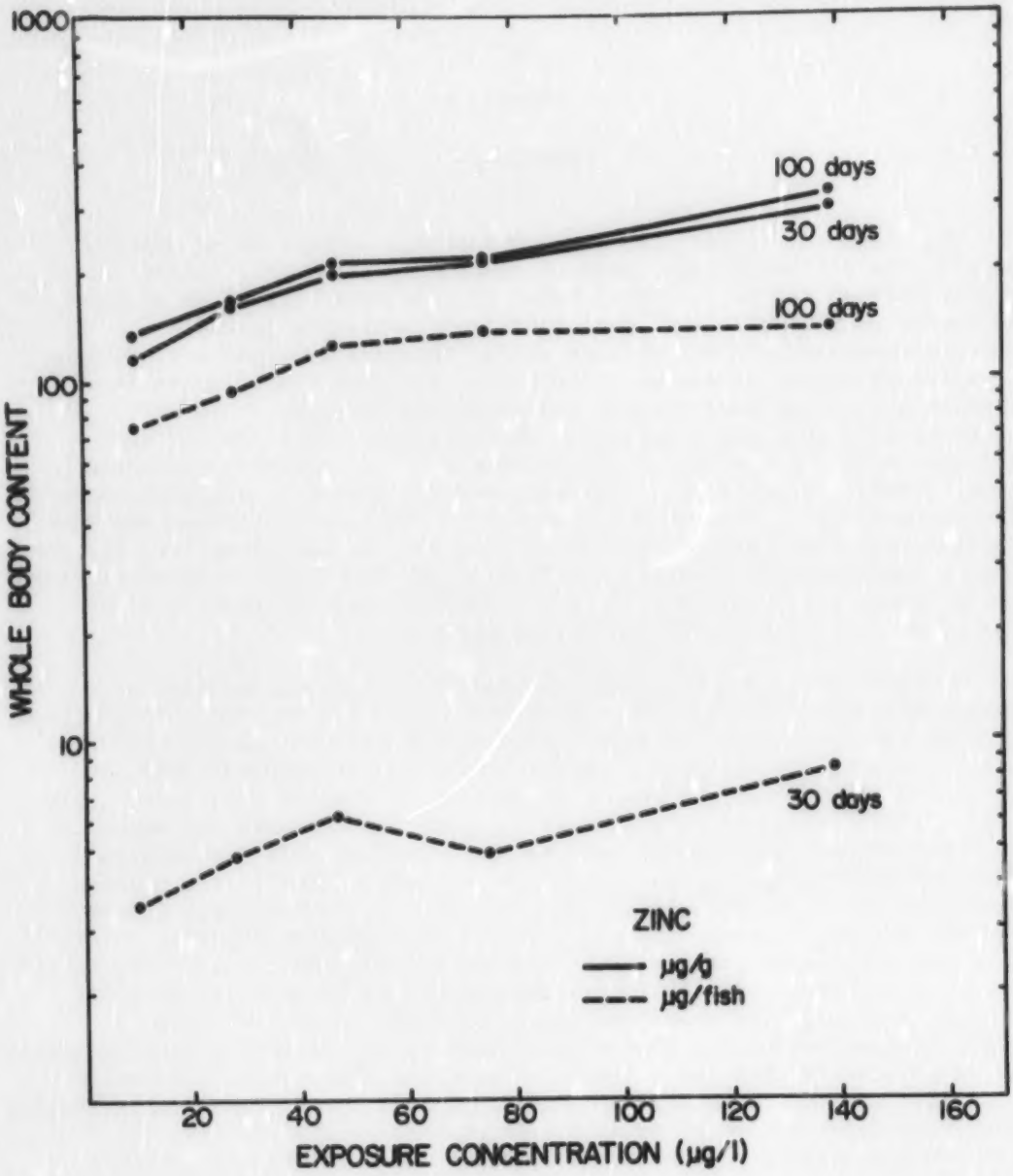

Figure 2. Log zinc content measured from whole body tissue of flagfish exposed to several zinc concentrations for 30 and 100 days.

SECTION 6

DISCUSSION

The response of this fish to sublethal concentrations of cadmium and zinc was different from those of other tested species. Pickering and Gast (1972) and Eaton (1974) found that embryos of fathead minnows and larvae of bluegills were the stages most sensitive to cadmium exposure. However, in the present study, spawning and embryo production was the most sensitive measure of effect. For zinc tests, survival of flagfish larvae and adult growth was adversely affected at lower concentrations than those affecting reproduction. This finding was different than the results of fathead minnow zinc exposures conducted by Brungs (1969). He found that reproduction was the most sensitive measure of zinc toxicity. Although the response of flagfish to cadmium and zinc was different from that of exposed fathead minnows and bluegills, the present results indicate that flagfish sensitivity was similar to that of cadmium exposed brook trout (Benoit, in press) and zinc exposed rainbow trout (Sinley et al. 1974) tested in soft water.

In this test, larvae previously exposed to cadmium and zinc as embryos were more tolerant than those not previously exposed. This effect was much more pronounced in zinc tests as indicated by results shown in Tables 2 and 3. Sinley et al. (1974) reported that rainbow trout not exposed to zinc as embryos may be as much as four times more susceptible to zinc than fish previously exposed as embryos. Although the mechanism for this phenomenon is poorly understood, Wedemeyer (1968) showed that coho salmon (Oncorhynchus kisutch) eggs accumulate zinc to the highest proportion in the chorion (70%), but that the developing embryos only accumulate a small amount (1%). Wedemeyer also pointed out that malachite green concentrations greater than 5 ppm increased zinc permeability of the vitelline membrane, thereby increasing the uptake of zinc in the yolk. Although the malachite green concentrations used to control fungal growth in the present tests were lower than those increasing zinc permeability, the danger exists that the toxicity of zinc was affected to some extent. No attempt was made to study the effects of this dye on zinc uptake in this test. The known action of malachite green on zinc and possibly other pollutants suggests, however, that it not be used in toxicity studies.

Relationships between cadmium tissue uptake and water concentration in this test were similar to those observed by Mount and Stephan (1967a) for cadmium-exposed bluegills. The leveling off of cadmium uptake at 16 µg/liter indicate that a possible equilibrium was reached between cadmium concentrations in the water and in the tissues. Comparisons between 30- and 100-day exposed fish and accumulations expressed on a

concentration (µg/gram) and total uptake (µg/fish) basis (Figure 1) show that cadmium accumulated with age, but at a rate that was slower than the growth of the fish. Similar results were indicated for the uptake of zinc (Figure 2). The absence of significant additional metal uptake on a concentration basis was probably related to mechanisms affecting rates of uptake and elimination as was proposed by Coleman and Cearley (1974) for silver uptake in largemouth bass (Micropterus salmoides) and bluegills. Additionally, tissue concentration did not differ significantly between fish exposed to zinc as embryos and those not previously exposed. This was attributed to the inability of zinc to penetrate the egg chorion to a large extent as was discussed by Wedemeyer (1968). Similar tissue concentrations in parents and progeny were probably the result of the same phenomenon. Experiments involving embryo and larval analyses would help to clarify mechanisms of metal uptake.

Abnormal behavior of exposed fish occurred at cadmium and zinc concentrations that caused death and inhibited reproduction. Cadmium especially had this effect on male fish during spawning. Eaton (1974) and Benoit et al. (in press) saw similar behavior in cadmium-exposed bluegills and brook trout. Abnormal behavior of bass and bluegills (uncontrolled swimming movements, convulsions, loss of equilibrium, and apparent coma) was also observed by Cearley (1971) when these fish were exposed to cadmium and silver. This type of behavior was attributed to the inhibition of acetylcholinesterase, causing death by paralysis of the muscles of respiration and/or depression of the respiratory center.

The flagfish application factors reported here for cadmium and zinc were similar to those reported by Eaton (1974) and Brungs (1969) for cadmium-exposed bluegills and zinc-exposed fathead minnows, respectively. Since both bluegill and fathead minnow tests were conducted in hard water compared to the soft water of this test, it would appear that water hardness, at least for these tests would not affect the estimation of chronic safe concentrations for these metals on the basis of calculated application factors.

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APPENDICES

		Page
A	Recommended Bioassay Procedures for *Jordanella floridae* (Goode and Bean) Chronic Tests	19
B	Cadmium Concentrations in Whole Fish Tissue After 30 and 100 Days of Exposure	32
C	Zinc Concentrations in Whole Fish Tissue After 30 and 100 Days of Exposure (test 1 begun with larvae exposed as embryos; test 2 begun with unexposed larvae)	33

APPENDIX A

RECOMMENDED BIOASSAY PROCEDURE FOR

JORDANELLA FLORIDAE (GOODE AND BEAN) CHRONIC TESTS

A. Physical system

1. Diluter: Proportional diluters (Mount and Brungs, 1967) should be employed for all long-term exposures. Check the operation of the diluter daily, either directly or through measurement of toxicant concentrations. A minimum of five toxicant concentrations and one control should be used for each test with a dilution factor of not less than 0.30. An automatically triggered emergency aeration and alarm system must be installed to alert staff in case of diluter, temperature-control, or water-supply failure.

2. Toxicant mixing: A container to promote mixing of toxicant-bearing and w-cell water should be used between diluter and tanks. Separate delivery tubes should run from this container to each of the duplicate spawning and corresponding progeny tanks for a total of four delivery tubes for each concentration and the control.

3. Tank: Duplicate spawning tanks should be made of glass or a combination of glass and stainless steel. They should measure 30- x 60- x 30-cm. Separate progeny tanks (one corresponding to each spawning tank) are of the same kind and size. Test water is to be supplied by delivery tubes from the mixing cells described in step 2 above.

Water depth in tanks should be 23-cm.

4. Flow rate: The flow rate of each tank (spawning or progeny) should be equal to 6-10 tank volumes/24 hr.

5. Aeration: Total dissolved oxygen levels should never be allowed to drop below 60% saturation, and flow rates must be increased if oxygen levels to drop below 60%. As a first alternative flow rates may be increased above those specified in A.4. Only aerate (with oil-free air) if testing a non-volatile toxic agent, and then as a last resort, to maintain dissolved oxygen at 60% of saturation.

6. Cleaning: All spawning tanks and progeny tanks after larvae swim up must be siphoned a minimum of two times weekly and brushed or scraped when algal or fungus growth becomes excessive.

7. Spawning substrate: Substrates are made of orlon yarn, preboiled to remove excess dye, strung on a 10- x 15-cm frame of stainless steel. The yarn is strung so that the strands are parallel with little or no space between strands. Any dark-colored yarn in satisfactory. White and bright colors, such as yellow and orange, are not accepted by the fish. Smooth-surfaced line, such as braided nylon or monofilament, induces egg eating by adults and is therefore not recommended.

8. Egg cup: Egg-incubation cups are made from 4-oz, 5-cm OD round glass jars with the bottoms cut off. One end of the jar is covered with stainless steel or nylon screen (with a minimum of 16 meshes per cm). Cups are oscillated in the test water by means of a rocker-arm apparatus driven by a 4-5 rpm electric motor (Mount, 1968). The vertical-travel distance of the cups should be 2.5-3.9-cm.

9. Light: The Duro-Test Vita-lite[1],[2] lights used should simulate sunlight as nearly as possible. Fluorescent tubes have proved satisfactory at the NWQL.

10. Photoperiod: A constant photoperiod of 16 hr light and 8 hr darkness should be maintained throughout the entire test. Gradual changes in light intensity at dawn and dusk (Drummond and Dawson, 1970) are desirable as a sudden flood of light is a shock to the fish. Any gradual changes of light intensity should be included in the photoperiod and should not last for more than 1/2 hr from full on to full off and vice versa.

11. Temperature: Temperature should not deviate instantaneously from 25° C by more than 2° C and should not remain outside the range of 24°-26° C for more than 48 hr at a time. Temperature should be recorded continuously.

12. Disturbance: Adults and larvae should be shielded from disturbances, such as people continually walking past the tanks, or from extraneous lights that might alter the intended photoperiod.

13. Construction materials: Construction materials that contact the diluent water should not contain leachable substances and should not sorb significant amounts of substances from the water. Stainless steel is probably the preferred construction material. Glass absorbs some trace organic compounds significantly. Rubber should not be used. Plastic containing fillers, additives,

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[1]Mention of trade names does not constitute endorsement.

[2]Duro-Test, Inc., Hammond, Ind.

stabilizers, plasticizers, etc., should not be used. Teflon, nylon, and their equivalents do not contain leachable materials and do not sorb significant amounts of most substances. Unplasticized polyethylene and polypropylene do not contain leachable substances, but may sorb very significant amounts of trace organic compounds.

14. Water: The water used should be from a well or spring if at all possible, or alternatively from a surface water source. Only as a last resort should water from a chlorinated municipal water supply be used. If the water supply is conceivably contaminated with fish pathogens, the water should be passed through an ultraviolet or similar sterilizer immediately before it enters the test system.

B. Biological system

1. Test animals: Obtain original stock of flagfish from commercial Florida supplier. The original fish should not be used as test animals but only to initiate a laboratory stock. Use only F_1 or later generations for testing. Groups of starting fish should contain a mixture of approximately equal numbers of eggs or larvae from at least three different females. Set aside enough eggs or larvae at the start of the test to supply an adequate number of fish for the acute mortality bioassays used in determining application factors.

2. Beginning test: Distribute 40-50 eggs or twenty 5- to 7-day-old larvae per duplicate tank by using a stratified random assignment (see D.3.). Extra test animals may be added at the beginning so that fish can be removed periodically for special examinations or for residue analysis.

3. Food: All fish over approximately 2.5 cm long should be fed a basic diet of frozen adult brine shrimp ad libitum at least twice daily supplemented by one daily feeding with a high quality fine granule dry trout food. Check for pesticides in each lot. It is recommended that rapidly growing fish, between the ages of 1 week and 8 weeks, be fed as much as they will eat 6-8 times daily. For 2-4 days following hatching, the larvae should be fed a flour-fine dry food. If zooplankton is available it is a superior ration. At 2-4 days of age newly hatched brine shrimp nauplii should be given as a basis of the larval diet.

4. Disease: Handle disease outbreaks according to their nature; all tanks should receive the same treatment whether all contain sick fish or not. Hold the frequency of treatment to a minimum.

5. Measuring fish: Record the length and weight of individual fish discarded at 30 days as a result of thinning (see B.6.) and of growth-study fish at termination time of 30 days.

6. Thinning: When the starting fish are 30 days old, randomly reduce the number of surviving fish in each tank to 15. Record the number of discarded fish per tank. When fish are 5 weeks old, place a spawning substrate at each end of the tank. When a male becomes territorial over each substrate in a tank remove the extra males and randomly remove all females but five.

7. Removing eggs: Remove eggs from spawning substrates starting at the same time each day in mid-afternoon. When a substrate is removed from a tank it should immediately be replaced with a clean substitute. Upon removal from a tank the substrate is immersed in a shallow glass or stainless steel pan in water dipped from the source tank. With a strong light over the work area, each yarn

strand may be checked for eggs which are removed with a propipettor. Eggs should be counted and then either retained for incubation or discarded.

8. Egg incubation and larval selection: Impartially select 50 unbroken eggs from spawnings of 50 eggs or more and place them in an egg incubator cup for determining viability and hatchability. Count the remaining eggs and discard them. Viability and hatchability determinations are made on the first four spawnings and then on alternate spawnings until termination of this portion of the study, 45 days following the first spawning of 30 or more eggs in any tank on any one substrate. If fewer than 50 eggs are present on any substrate 3 days after the first production of 30 or more eggs on one substrate, it is advisable to hatch those that are present.

   When larvae begin to hatch, on the 4th day or later depending upon toxicant effect, they should not be handled again or removed from the cups until all have hatched. At this time transfer 20 larvae to each corresponding progeny tank. If fewer than 20 larvae are present in any tank, transfer the number present. Entire egg-cup groups not used for survival and growth studies should be counted and discarded.

9. Progeny transfer: Additional important information on hatchability and larval survival is to be gained by transferring control eggs immediately after spawning to concentrations where spawning is reduced or absent, or to where an affect is seen on survival of eggs or larvae, and by transferring eggs from these concentrations to the control tanks.

10. Larval exposure: From early spawnings in each duplicate tank, use the larvae hatched in the egg incubator cups (Step B.8. above) for 30-day growth and survival exposures in the progeny tanks. At least one group of progeny from each adult tank should be reared for 30 days in the corresponding progeny tank, but another 30-day growth study can be conducted with larvae transferred from a different, non-corresponding spawning tank. Other alternatives are to conduct only a single 30-day exposure in each progeny tank, or to conduct two consecutive exposures with progeny from the corresponding spawning tank. Plan ahead in setting up eggs for hatchability so that a second group of larvae is ready to be tested for 30 days as soon as possible after the previously tested group comes out of the progeny tanks. Record mortalities and total lengths and weights of larvae at 30 days. No fish (larvae, juveniles, or adults) should be fed for 24 hr before weighing.

11. Parental termination: Parental fish testing should be terminated at the end of the 45th day or the beginning of the 46th day after the first production in any tank of 30 or more eggs on one spawning substrate. Record sex, total length, and total weight of each fish.

12. Special examinations: Fish and eggs obtained from the test should be subjected to physiological, biochemical, histological, and other examinations that may indicate certain toxicant-related effects.

13. Necessary data: The following data must be reported for each tank of a chronic test:

    a. Number of normal and deformed fish at 30 days, and total length, weight, and number of either sex at termination of test;

b. Mortality during the test;

c. Number of spawns and eggs;

d. Hatchability; and

e. Larval survival, growth, and deformities.

C. Chemical system

1. Preparing a stock solution: If a toxicant cannot be introduced into the test water as is, a stock solution should be prepared by dissolving the toxicant in water or an organic solvent. Acetone has been the most widely used solvent, but dimethylformanide (DMF) and triethylene glycol may be preferred in many cases. If none of these solvents is acceptable, other water-miscible solvents, such as methanol, ethanol, isopropoanol, acetonitrile, dimethylacetamide (DMAC), 2-ethoxyethanol, glyme (dimethylether of ethylene glycol, diglyme (dimethyl ether of diethylene glycol), and propylene glycol, should be considered. However, dimethyl sulfoxide (DMSO) should not be used because of its biological properties.

Problems of rate of solubilization or solubility limit should be solved by mechanical means if at all possible. Solvents, or, as a last resort, surfactants, can be used for this purpose only after they have been proven to be necessary in the actual test system. The suggested surfactant is p-tert-octylphenoxynonaethoxy-ethanol (p-1, 1, 3, 3-tetramethylburylphenoxynonaethoxyethanol, OPE_{10}) (Triton X-100, a product of the Rohm and Haas Company), or equivalent.

The use of solvents, surfactants, or other additives should be avoided whenever possible. If an additive is necessary, reagent

grade or better should be used. The amount of an additive used should be kept to a minimum, but the calculated concentration of a solvent to which any test organisms are exposed must never exceed one one-thousandth of the 96-hr TL50 for test species under the test conditions and must never exceed 1 g/l. of water. The calculated concentration of surfactant or other additive to which any test organisms are exposed must never exceed one-twentieth of the concentration of the toxicant and must never exceed 0.1 g/l. of water. If any additive is used, two sets of controls must be used, one exposed to no additives and one exposed to the highest level of additives to which any other organisms in the test are exposed.

2. Measurement of toxicant concentration: As a minimum the concentration of toxicant must be measured in one tank at each toxicant concentration every week for each set of duplicate tanks, alternating tanks at each concentration from week to week. Water samples should be taken about midway between the top and bottom and the sides of the tank and should not include any surface scum or material stirred up from the bottom or sides of the tank. Equivolume daily grab samples can be composited for a week if it has been shown that the results of the analysis are not affected by storage of the sample.

   Enough grouped grab samples should be analyzed periodically throughout the test to determine whether or not the concentration of toxicant is reasonably constant from day to day in one tank and from one tank to its duplicate. If not, enough samples must be analyzed weekly throughout the test to show the variability of the toxicant concentration.

3. Measurement of other variables: Temperature must be recorded continuously (See A.11.).

Dissolved oxygen must be measured in the tanks daily, at least 5 days a week on an alternating basis, so that each tank is analyzed once each week. However, if the toxicant or an additive causes a depression in dissolved oxygen, the toxicant concentration with the lowest dissolved oxygen concentration must be analyzed daily in addition to the above requirement.

A control and one test concentration must be analyzed weekly for pH, alkalinity, hardness, acidity, and conductance, or more often, if necessary, to show the variability in the test water. However, if any of these characteristics are affected by the toxicant, the tanks must be analyzed for that characteristic daily, at least 5 days a week, on an alternating basis so that each tank is analyzed once every other week.

At a minimum, the test water must be analyzed at the beginning and near the middle of the test for calcium, magnesium, sodium, potassium, chloride, sulfate, total solids, and total dissolved solids.

4. Residue analysis: When possible and deemed necessary, mature fish, and possibly eggs, larvae, and juveniles, obtained from the test should be analyzed for toxicant residues. For fish, muscle should be analyzed, and gill, blood, brain, liver, bone, kidney, GI tract, gonad, and skin should be considered for analysis. Analyses of whole organisms may be done in addition to, but should not be done in place of, analyses of individual tissues, especially muscle.

5. Methods: When they will provide the desired information with acceptable precision and accuracy, methods described in Methods for Chemical Analysis of Water and Wastes (U.S. Environmental

Protection Agency, 1971) should be used unless another method requires much less time and can provide the desired information with the same or better precision and accuracy. At a minimum, accuracy should be measured by using the method of known additions for all analytical methods for toxicants. If available, reference samples should be analyzed periodically for each analytical method.

D. Statistics

1. Duplicates: Use true duplicates for each level of toxic agent, i.e., no water connections between duplicate tanks.

2. Distribution of tanks: The tanks should be assigned to locations by stratified random assignment (random assignment of one tank for each level of toxic agent in a row followed by random assignment of the second tank for each level of toxic agent in another or an extension of the same row).

3. Distribution of test organisms: The test organisms should be assigned to tanks by stratified random assignment (random assignment of one test organism to each tank, random assignment of a second test organism to each tank, etc.).

E. Miscellaneous

1. Fungusing of eggs: If fungusing of viable eggs occurs during incubation it may be necessary to use a chemical fungicide. One successful treatment with no apparent ill effects has been a daily 5-min dip of eggs within each egg cup in Malachite green. Prepare a stock solution of 2 Malachite green per liter of water and retain this for use throughout the test. For treatment of the eggs, add 0.5 ml of stock solution to 250 ml water at test temperature and immerse each egg cup in the dip for a period of 5 min and then replace on the rocker. Opaque-white and fungused

eggs should be removed daily from each egg cup. Should algal growth or detritus foul the egg-cup screen, preventing free flow of water during the rocking cycle, the screens must be cleaned taking care not to damage the eggs. If flow is free, do not disturb the eggs.

2. Additional information: All routine bioassay flow-through methods not covered in this procedure (e.g., physical and chemical determinations, handling of fish) should be followed as described in Standard Methods for the Examination of Water and Wastewater (American Public Health Association, 1971), or information may be requested from appropriate persons at Duluth or Newtown.

3. Acknowledgments: These procedures for Jordanella floridae were compiled by Wesley Smith for the Committee on Aquatic Bioassays.

4. References: For additional information concerning the flagfish and continuous-flow bioassay testing, the following references are listed:

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Approved by the Committee
on Aquatic Bioassays, NWQL

Approved by the Director, NWQL

APPENDIX B

CADMIUM CONCENTRATIONS IN WHOLE FISH TISSUE AFTER 30 AND 100 DAYS OF EXPOSURE

Measured cadmium concentration in water (μg/l.)	Number of samples	μg/g Mean ± 1 standard deviation	Total μg/fish Mean ± 1 standard deviation
		30-day exposure	
31	6	74.0±29.4	1.1±0.2
16	10	70.8±18.6	2.0±1.0
8.1	10	39.3±11.0	1.2±0.5
4.1	10	26.9±9.8	0.9±0.5
1.7	10	16.9±6.0	0.5±0.2
0.11 (Control)	10	1.7±0.6	0.1±0.01
		100-day exposure (adult)	
31	1	34.1	4.4
16	4	21.1±8.5	3.7±1.3
8.1	10	10.4±2.4	6.5±2.0
4.1	10	6.0±1.6	4.5±1.6
1.7	10	4.1±1.3	2.3±0.5
0.11 (Control)	10	0.2±0.1	0.2±0.01
		30-day exposure (F_1)	
31	-	-	-
16	10	30.0±5.2	0.8±0.2
8.1	10	26.7±7.4	1.3±1.6
4.1	10	18.4±4.1	0.6±0.2
1.7	10	9.9±2.3	0.4±0.1
0.11 (Control)	10	-[a]	-[a]

[a]No detection of cadmium in fish tissue.

APPENDIX C

ZINC CONCENTRATIONS IN WHOLE FISH TISSUE AFTER 30 AND 100 DAYS OF EXPOSURE (test 1 begun with larvae exposed as embryos; test 2 begun with unexposed larvae)

Measured zinc concentration in water (µg/l.)	Test 1 µg/g Mean ± standard deviation[b]	Test 1 Total µg/fish Mean ± standard deviation	Measured zinc concentration in water (µg/l.)	Test 2 µg/g Mean ± standard deviation[b]	Test 2 Total µg/fish Mean ± standard deviation
	30-day exposure			30-day exposure	
267	-[a]	-	267	-[a]	-
139	293±55.3	8.3±4.0	139	-	-
75	211±32.4	4.9±1.5	85	-	-
47	196±34.0	6.2±3.0	51	228±13.0	11.1±5.2
28	160±26.9	4.4±2.2	26	178±22.7	7.1±2.4
10 (Control)	116±29.1	3.5±1.5	0 (Control)	147±19.4	8.6±4.4
	100-day exposure (adult)			100-day exposure (adult)	
267	-	-	267	-	-
139	324±18.7	137±87.5	139	-	-
75	214±41.3	137±52.6	85	235±13.7	155±87.9
47	212±26.6	128±47.7	51	211±20.7	110±29.9
28	167±29.2	94±23.2	26	174±28.9	86.6±42.4
10 (Control)	134±29.4	75±30.5	0 (Control)	117±6.1	56.8±19.6
	30-day exposure (F_1)			30-day exposure (F_1)	
267	-	-	267	-	-
139	272±34.9	12.5±5.7	139	-	-
75	220±58.7	8.5±7.8	85	200±10.0	4.4±1.2
47	205±55.9	5.2±1.6	51	178±12.5	6.4±1.6
28	157±24.1	3.7±0.8	26	161±15.0	4.4±1.7
10 (Control)	115±7.5	3.5±1.3	0 (Control)	145±15.5	4.9±1.7

[a] No fish were analyzed because of high mortality.

[b] Five fish were analyzed per concentration.

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16. ABSTRACT

Cadmium and zinc toxicity to the flagfish (Jordanella floridae) was determined on the basis of 96-hr median lethal concentrations (LC50) and significant decreases ($P = 0.05$) in survival, growth, and reproduction over the complete life cycle of the fish. The 96-hr LC50 values for cadmium and zinc to juvenile flagfish were 2,500 and 1,500 µg/liter, respectively. In chronic tests, reproduction was the most sensitive indicator of cadmium toxicity and was inhibited at 8.1 µg/liter. Tissue-concentration analysis showed that fish exposed to concentrations of 1.7 µg/liter and above accumulated significantly greater amounts of cadmium than those in the controls. In zinc tests, survival of larvae (not exposed as embryos) and growth of females were the most sensitive measure of zinc toxicity and were reduced at respective concentrations of 85 and 51 µg/liter. Significant uptake of zinc occurred in fish exposed to concentrations of 47 µg/liter and above. The lowest cadmium and zinc concentrations causing adverse effects to the flagfish were similar to those affecting other fish species. Application factors for both metals were similar to those reported for cadmium exposed bluegills (Lepomis macrochirus) and zinc exposed fathead minnows (Pimephales promelas) in hard water.

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17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Water pollution Metals Toxicity Contamination Fishes Effluents Fresh water Fresh water fishes Cadmium Zinc	Heavy metals Flagfish Toxicity tests Acute effects Chronic effects Flow-through	6A 6F

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